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Genetic Control of Amadori Product Degradation in *Bacillus subtilis* via Regulation of *frlBONMD* Expression by FrlR▽†

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***Bacillus subtilis* is capable of degrading fructosamines. The phosphorylation and the cleavage of the resulting fructosamine 6-phosphates is catalyzed by the *frlD* and *frlB* gene products, respectively. This study addresses the physiological importance of the *frlBONMD* genes (formerly *yurPONML*), revealing the necessity of their expression for growth on fructosamines and focusing on the complex regulation of the corresponding transcription unit. In addition to the known regulation by the global transcriptional regulator CodY, the *frl* genes are repressed by the convergently transcribed FrlR (formerly YurK). The latter causes repression during growth on substrates other than fructosamines. Additionally, we identified in the first intergenic region of the operon an FrlR binding site which is centrally located within a 38-bp perfect palindromic sequence. There is genetic evidence that this sequence, in combination with FrlR, contributes to the remarkable decrease in the transcription downstream of the first gene of the *frl* operon.**

Amadori products (fructosamines), the first stable intermediates of the Maillard reaction, result from nonenzymatic glycation of amino acids or proteins with reducing sugars such as glucose. As Amadori products are found in heated food and cause several diseases in connection with diabetes and aging (4), enzymes catalyzing the degradation of Amadori products are of interest to industry and medicine for food processing and diagnostic purposes.

There are two enzymatic mechanisms for the deglycation of Amadori products (reviewed in reference 7): fructosyl-amino acid oxidases (known as Amadoriases) and fructosamine kinases. The former deglycate by means of oxidation, generating the corresponding amino acid, glucosone, and H_2O_2. Amadoriases have been found in *Aspergillus* and *Penicillium* spp. (38, 42) but also in bacteria such as *Arthrobacter* and *Corynebacterium* spp. (9, 32). Fructosamine kinases phosphorylate the Amadori products prior to cleavage. Mammalian enzymes add the phosphate at C-3, and the product subsequently undergoes autocatalytic degradation (20). However, *Escherichia coli* and *Bacillus subtilis* kinases phosphorylate at C-6; further processing of such intermediates needs a second deglycase enzyme catalyzing the cleavage of the fructosamine 6-phosphates to generate, for example, glucose 6-phosphate and a free amine.

E. coli grows on fructosyl-lysine as the sole carbon and nitrogen source, and expression of *frlD* (encoding the kinase) and *frlB* (encoding the deglycating protein) is induced by fructosyl-lysine (39). In *B. subtilis*, *frlD* and *frlB* belong to a gene cluster which additionally comprises three genes coding for putative transporters (*frlONM*) as well as the convergently oriented putative repressor gene *frlR* (Fig. 1). Substrate specificities of FrlD and FrlB are quite different than those of the enzymes of *E. coli*, as *B. subtilis* acts on α-glycated amino acids rather than on ε-glycated lysine (catalytic efficiencies >30-fold higher), the latter being the preferred substrate of the *E. coli* enzymes (40). However, other than the biochemical function of the enzymes, little is known about the encoding genes in *B. subtilis*; neither their physiological significance nor their regulation has been studied in detail.

The promoter upstream of the first open reading frame, *frlB*, was identified as responsive to CodY (26), which is a global transcriptional regulator that controls the expression of more than 100 mostly late-exponential-phase genes in *B. subtilis*, predominantly as a repressor (reviewed in reference 34). The direct interaction of CodY with the *frlB* promoter and a strong derepression in a *codY* deletion strain was revealed (3).

With the recent revision of the *B. subtilis* 168 genome annotation, a putative regulator gene, *frlR*, with similarities to the respective gene in *E. coli*, was identified (2). The predicted FrlR protein was classified as a member of the GntR superfamily, which includes more than 8,500 proteins widely distributed throughout bacteria (reviewed in reference 17). At their conserved N termini, GntRs display helix-turn-helix (HTH) domains; the C terminus routinely facilitates effector binding and/or oligomerization (14). GntRs control a number of fundamental processes, such as catabolism, anabolism, motility, development, antibiotic production, and virulence (5, 8, 12, 15, 18, 19). Based on differences within the C termini and the recognition sequences, seven GntR subfamilies (FadR, HutC, MocR, YtrA, DevA, PlmA, and AraR) have been defined. According to the crystal structure, FrlR belongs to the HutC subfamily (28).

In this study, we investigated the physiological importance of the *frl* operon in *Bacillus subtilis* and inspected its regulation by FrlR.

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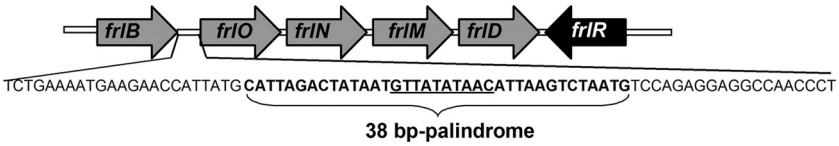

FIG. 1. Schematic illustration of the *frl* operon with the *frlB* and *frlO* intergenic region. The 38-bp perfect palindromic sequence is shown in bold letters. A putative FrlR binding site (underlined) is located in the center of the intergenic region.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. *E. coli* XL10-Gold (Stratagene, La Jolla, CA) was used for routine cloning, and *E. coli* BL21 (37) was used for heterologous protein expression. *E. coli* strains were grown in Luria-Bertani (LB) broth (33). *B. subtilis* strains were grown in LB medium or M9 medium supplemented with 1 mg/ml Trp with the appropriate carbon and nitrogen sources. For cultivation in Amadori product, medium M9 was supplemented with 30% (vol/vol) of a 7.5% (wt/vol) Amadori crude preparation (see below). In control medium (M9-Glc-NH_4), the equivalent amount of glucose (7.5 g/liter) and, as a nitrogen source, NH_4Cl (2.5 g/liter) were used. As a second control medium, the equivalent amount of arginine was used as the sole carbon and nitrogen source (17.7 g/liter). The growth was monitored by measuring the optical density at 600 nm (OD_{600}). When required, the following antibiotics were added with the indicated final concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml in *E. coli* and 7.5 μg/ml in *B. subtilis*; erythromycin, 0.5 μg/ml; chloramphenicol, 0.5 μg/ml; tetracycline, 15 μg/ml; and phleomycin, 1 μg/ml.

Construction of expression vectors. Plasmids and oligonucleotides used in this study are listed in Table 2 and Table S1 in the supplemental material, respectively. Standard procedures were used for DNA work (33).

For amplification of *codY* and *frlR* from *B. subtilis* 168 DNA, the primer pairs codY_NdeI_for/codY_XhoI_rev and frlR_NdeI_for/frlR_XhoI_rev were used and inserted into XhoI/NdeI-digested pET26b$^+$.

The *Bacillus* expression vector pVD28 was constructed by amplification of the vector backbone of a pBC16 derivative with pVD19_EcoR_for and pVD19_XhoI_rev and amplification of the expression cassette from pHT01 by using the primers Pgrac_EcoRI_for and Pgrac_XhoI_rev. The amplification products were cut with EcoRI and XhoI and subsequently ligated.

Construction of *B. subtilis* knockout mutants. For the deletion of genes in *B. subtilis*, flanking regions of the respective genes were fused to an antibiotic resistance gene by overlap extension PCR (16), and the resulting cassette was cloned into pUC18. The obtained vectors were introduced into *B. subtilis* 168 by means of natural competence. Double recombination events via the flanks generated mutants selectable by antibiotic resistance; mutant genotypes were confirmed by PCR analysis and sequencing.

For replacement of *frlR*, the flanks were amplified by using the primers FlA-KpnV/FlA_SOE_Erm and FlB_yurK_EcoR/Erm SOE FlB_yurK and the erythromycin resistance gene with the primers FlA_SOE_Erm/FlB_yurK_SOE_Erm. For disruption of *codY*, the flanking regions were amplified with primer pairs CodY_FlA-KpnV/Kan_SOE_FlA_codY and CodY_FlB_EcoR/Kan SOE FlB_codY and a kanamycin resistance gene with the primers FlA_codY_SOE_Kan/FlB_codY_SOE_Kan. For deletion of the entire *frlBONMD* operon along with the regulator gene *frlR*, homologous regions were amplified by using the primers FlA-KpnV/Erm_SOE_FlA and FlB_EcoR/Erm SOE FlB. The erythromycin resistance gene was amplified using the primers FlA_SOE_Erm and FlB_SOE_Erm. To enable proper β-galactosidase reporter gene assays, the encoding *lacA* gene was deleted by using primer pairs LacA_FlA-KpnV/ble_SOE_FlA_lacA and FlB LacA_EcoR/ble SOE FlB_lacA for the flanks and FlA_lacA_SOE_ble/FlB_lacA_SOE_ble for the bleomycin resistance gene.

Construction of promoter test strains. The promoter fragments were amplified while concomitantly generating EcoRI and BamHI restriction sites and cloned into EcoRI/BamHI-digested pDH32M (22). For P_{frlB} amplification, the primers P-frlB-EcoRI_for/Pfrl_pMUTIN_BamHI_rev were used. P_{frlR} was amplified with P-frlR-EcoRI_for/P-frlR-BamHI_rev and P_{hpaII} by using PhpaII-BamHI_for/PhpaII_SnaBI_rev. For the amplification of the elongated promoter fragments of P_{frlB}, the *frlB* gene, and the *frlB-frlO* intergenic region ($I_{frlB\text{-}frlO}$), the primer pair P-frlB-EcoRI_for/P-frlO-BamHI_rev was used. The construction of the elongated P_{hpaII} with the *frlB* gene and $I_{frlB\text{-}frlO}$, PCR was carried out using the primer pairs PhpaII-BamHI_for/frlB_SOE_PhpaII and PfrlO SnaBI_rev/PhpaII_SOE_frlB, and a fusion of the amplification products was carried out using overlap extension PCR (16).

After introducing the DNA by means of natural competence, the promoter

TABLE 1. Strains used in the study

Strain	Genotype[a]	Source or reference
E. coli		
XL10-Gold	*endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac* Hte Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173* Tet^r [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r Amy Cm^r)]	Stratagene
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	37
B. subtilis		
168	*trpC2*	Laboratory stock
DE01	*trpC2* Δ*frlBONMD* Δ*frlR*::Erm^r	This work
DE02	*trpC2* Δ*frlR*::Erm^r	This work
DE11	*trpC2* Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlB}-*lacZ*) Cat^r]	This work
DE12	*trpC2* Δ*frlR*::Erm^r Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlB}-*lacZ*) Cat^r]	This work
DE13	*trpC2* Δ*codY*::Kan^r Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlB}-*lacZ*) Cat^r]	This work
DE14	*trpC2* Δ*frlR*::Erm^r Δ*codY*::Kan^r Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlB}-*lacZ*) Cat^r]	This work
DE16	*trpC2* Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlR}-*lacZ*) Cat^r]	This work
DE20	*trpC2* Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlB}-*frlB*-$I_{frlB\text{-}frlO}$-*lacZ*) Cat^r]	This work
DE21	*trpC2* Δ*frlR*::Erm^r Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlR}-*lacZ*) Cat^r]	This work
DE22	*trpC2* Δ*frlR*::Erm^r Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlB}-*frlB*-$I_{frlB\text{-}frlO}$-*lacZ*) Cat^r]	This work
SK1	*trpC2* Δ*lacA*::Ble^r *amyE*::[Δ(P_{hpaII}-*frlB*-$I_{frlB\text{-}frlO}$-*lacZ*) Cat^r]	This work
SK2	*trpC2* Δ*lacA*::Ble^r *amyE*::[Δ(P_{hpaII}-*lacZ*) Cat^r]	This work
SK3	*trpC2* Δ*frlR*::Erm^r Δ*lacA*::Ble^r *amyE*::[Δ(P_{hpaII}-*frlB*-$I_{frlB\text{-}frlO}$-*lacZ*) Cat^r]	This work
SK4	*trpC2* Δ*frlR*::Erm^r Δ*lacA*::Ble^r *amyE*::[Δ(P_{hpaII}-*lacZ*) Cat^r]	This work
SK7	*trpC2* Δ*lacA*::Ble^r *amyE*::[Δ(P_{frlB}*-*lacZ*) Cat^r]	This work

[a] Amy, amylase expression.

TABLE 2. Plasmids used in the study

Plasmid	Description/genotype[a]	Source or reference
pUC18	Cloning vector (Amp^r)	41
pET26b$^+$	Vector enabling IPTG-inducible expression of gene of interest with 6× C-terminal His tag (Kan^r)	Novagen, Madison, WI
pDH32M	Integration vector for transcriptional fusions with *lacZ* at the *amyE* locus (Amp^r Cm^r)	22
pVD23	pUC18 derivative containing homologous regions upstream of *frlB* and downstream of *frlR* flanking the Erm^r gene (Amp^r Erm^r)	This work
pVD24	pUC18 derivative containing homologous regions upstream and downstream of *frlR* flanking the Erm^r gene (Amp^r Erm^r)	This work
pVD26	pUC18 derivative containing homologous regions upstream and downstream of *codY* flanking the Kan^r gene (Amp^r Kan^r)	This work
pVD28	Fusion of pBC16-vector backbone and IPTG-inducible expression cassette of pHT01 (Mobitec) (Tc^r)	This work
pVD30.1	pET26b$^+$ derivative containing *codY* gene (Kan^r)	This work
pSKL3	pET26b$^+$ derivative containing *frlR* gene (Kan^r)	This work
pVD33.1	pVD28 derivative containing *frlR* gene	This work
pVD34	pUC18 derivative containing homologous regions upstream and downstream of *lacA* flanking the Ble^r gene (Amp^r Ble^r)	This work
pVD36	pDH32M derivative containing the *frlB* promoter (Amp^r Cm^r)	This work
pVD37	pDH32M derivative containing the *frlR* promoter (Amp^r Cm^r)	This work
pVD39	pDH32M derivative containing the *frlB* promoter followed by the *frlB* gene and $I_{frlB\text{-}frlO}$ (Amp^r Cm^r)	This work
pSKL5	pDH32M derivative containing the *hpaII* promoter followed by the *frlB* gene and $I_{frlB\text{-}frlO}$ (Amp^r Cm^r)	This work
pSKL6	pDH32M derivative containing the *hpaII* promoter (Amp^r Cm^r)	This work

[a] IPTG, isopropyl-β-D-thiogalactopyranoside.

test mutants were first screened for chloramphenicol resistance and subsequently tested for an amylase-negative phenotype, as the promoter test cassettes were targeted to the *amyE* locus.

Transformation of *B. subtilis* by means of natural competence. *B. subtilis* cells were transformed by means of natural competence following a modified protocol (1); 500-μl aliquots of competent cells were mixed with various amounts of plasmid DNA (from 1 μg to 10 μg) and incubated for 30 min. For the regeneration of cells, 300 μl LB medium was added and incubated for 30 min. Cells were then plated on LB agar supplemented with the respective antibiotic.

Synthesis of Amadori products. Amadori products were synthesized by using a modified protocol (10). Glucose and arginine were mixed in a molar ratio of 1:2 in methanol and heated (65.5°C) for 10 to 14 h with stirring and continuous reflux cooling. Subsequently, the methanol was removed by using a rotary evaporator; the obtained product mixture was dissolved in double-distilled water to yield a final concentration of 7.5% (wt/vol).

Determination of glucose, arginine, and Amadori products. Glucose was determined with the glucose assay reagent of Sigma-Aldrich (St. Louis, MO) as suggested by the manufacturer.

Arginine was measured by ultraperformance liquid chromatography (UPLC) analysis with X-LC (Fasco, Gross-Umstadt, Germany). Arginine was derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and separated with a Gold Turbo Basic C_8 column (Fasco, Gross-Umstadt, Germany).

Relative amounts of Amadori products were measured with the GlyPro reagent of Genzyme Diagnostics (Cambridge, MA). Thus, Amadori products were quantified relative to an internal calibration standard provided with the kit, with a distinct concentration of glycated hemoglobin. Hence, the Amadori amount is specified as a "relative concentration," since it relates to the glycated hemoglobin control. Twenty-microliter volumes of each sample and of the calibration control (repeated at least twice) were added to 250 μl reagent 1 (R1) and incubated for 5 min at 37°C before measuring the OD_{550} (A1). Subsequently, 50 μl of reagent 2 (R2) was added, and after an additional 5 min at 37°C, the OD_{550} (A2) was measured. With the aid of the calibration control the (relative) Amadori product concentration (μmol/liter) was calculated by the equation $(\Delta A_{sample}/\Delta A_{calibrator}) \times 450$ μmol/liter, where $\Delta A = A2 - A1$.

RNA isolation and quantitative PCR (qPCR). *B. subtilis* 168 was cultivated in LB broth with the addition of 3% (wt/vol) Amadori product, and cells from 7 ml were harvested (4°C) during both exponential growth and stationary growth. Cell samples were added to 3.5 ml of precooled killing buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM NaN_3) and pelleted. After being washed with 5 ml lysis buffer (3 mM EDTA, 200 mM NaCl), the cells were resuspended in 700 μl lysis buffer and transferred into lysing matrix B tubes (Q-Biogene, Montreal, Quebec, Canada) containing 400 μl acidic phenol. Cell disruption was performed with the FastPrepTM FP 120 cell disrupter (BIO 101 Systems, Q-Biogene, Montreal, Quebec, Canada) for 35 s at 6.5 m/s. After centrifugation (12,000 × *g* for 30 min at 4°C), 350 μl of the upper phase was transferred into a fresh tube, and the RNA was purified with the aid of a KingFisher instrument (Thermo Electron Corporation; Cambridge, MA) and MagNa Pure LC RNA isolation kits (Roche Diagnostics AG, Rotkreuz, Switzerland) by applying the protocol suggested by the manufacturer. A DNA digestion step with RQ1 RNase-free DNase (Promega GmbH, Mannheim, Germany) was subsequently carried out, and the RNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The quality and purity of RNA were determined by using an Agilent 2100 bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) and an RNA 6000 NanoLab chip kit (Agilent Technologies Inc., Santa Clara, CA); total RNA was transcribed into cDNA by applying a high-capacity cDNA reverse transcription kit (Applied Biosystems, CA) with the protocol recommended by the manufacturer.

For the relative quantitative determination of *frlB* and *frlD* transcripts, qPCRs were performed in an MXPro 3500 system (Stratagene, La Jolla, CA) with the Brilliant SYBR green qPCR master mix kit (Stratagene, La Jolla, CA). A total of 0.032 ng cDNA was used per well, and the analysis was carried out in triplicate; the primers used for measurement of *frlB* and *frlD* transcripts were qPCR-yurP-2-for/qPCR-yurP-2-rev and qPCR-yurL-5-for/qPCR-yurL-5-rev, respectively. Primer design was done with the eprimer3 software (EMBOSS [the European Molecular Biology Open Software Suite] package). For the housekeeping gene *atpA*, which served as a reference, the primers qPCR-atpA-3-for/qPCR-atpA-3-rev were used. The software for the MXPro 3500 system (Stratagene, La Jolla, CA) was applied for the determination of threshold cycle (C_T) values, and ΔC_T values were calculated in relation to the value for *atpA*.

Expression and purification of FrlR and CodY proteins. *E. coli* BL21(DE3) expression strains containing pVD30.1 or pSKL3 in the pET26b$^+$ (Novagen, Madison, WI) plasmid including a C-terminally 6-histidine-tagged *codY* gene (pVD30.1) or *frlR* gene (pSKL3) were grown in autoinduction medium [50 mM $NaH_2PO_4 \cdot 1H_2O$, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.4% (wt/vol) glycerin, 3 mM glucose, 6 mM lactose, 10 g/liter NZ-Amine-AS, 5 g/liter Bacto yeast extract, 20 μM $CaCl_2 \cdot 2H_2O$, 10 μM $MnCl_2 \cdot 4H_2O$, 10 μM $ZnCl_2$, 2 μM $CoCl_2$, 2 μM $CuCl_2$, 2 μM $NiCl_2$, 2 μM $Na_2MoO_4 \cdot 2H_2O$, 2 μM Na_2SeO_3, 2 μM H_3BO_3, 50 μM $FeCl_3 \cdot 6H_2O$] supplemented with kanamycin (50 μg/ml). Cultivation was carried out at 30°C and 200 rpm (incubator ISF-1-W; Kühner, Birsfelden, Switzerland). After 24 h, cells were pelleted and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole), the cells were disrupted by sonication (amplitude of 40% for 2.5 min, with a time interval of 5 s and an S&M 0102 tube in a Sonics & Materials Inc. Vibra Cell [Meyrin/Satigny, Switzerland]), and the debris was removed by centrifugation. The 6×His-tagged CodY was purified using Ni-nitrilotriacetic acid (NTA) agarose obtained from Invitrogen (Darmstadt, Germany). The protein concentration in the eluates was measured using the Pierce bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL) by using bovine serum albumin (BSA) as a relative standard as recommended by the manufacturer.

Electrophoretic mobility shift assay (EMSA). Five femtomoles of 5′-digoxigenin (DIG)-labeled promoter fragments were incubated with increasing concentrations of C-terminally 6×His-tagged FrlR (FrlRcHis) in a 10-μl reaction mixture at 22°C for 20 min. The reaction mixture contained 20 mM Tris-HCl (pH 8.0), 50 mM sodium glutamate, 10 mM $MgCl_2$, 5 mM EDTA, 5% (vol/vol) glycerol, and 250 ng calf thymus DNA. Directly after incubation, 2 μl of a 5-times-concentrated sample buffer (18 mM Tris, 18 mM boric acid, 0.4 mM EDTA, 9% Ficoll type 400, 0.02% bromphenol blue) was added and the samples were loaded onto a 6% DNA retardation gel (Invitrogen, Darmstadt, Germany) running at 50 V and 4°C for 90 min. The DNA was electrotransferred (30 V for 90 min) to a positively charged nylon membrane (Porablot NY Plus nylon membrane, 0.45-μm pore size; Macherey-Nagel, Düren, Germany) using 0.5× Tris-borate-EDTA (TBE) as the transfer buffer. The immunological detection of the 5′-DIG-labeled promoter fragments was performed with an antidigoxigenin antibody (Roche, Mannheim, Germany) and nitroblue tetrazolium (NBT)-BCIP (5-bromo-4-chloro-3-indolylphosphate) solution as recommended by the manufacturer.

Promoter test analysis by *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay. Promoter test mutants were cultivated in 100-ml Erlenmeyer flasks (300 rpm in incubator ISF-1-W; Kühner, Birsfelden, Switzerland) (37°C) in a total volume of 10 ml medium. Reporter gene analyses were carried out as described previously (23) with the following equation: Miller units (MU) = $(OD_{420} \times 1{,}000)/(OD_{600} \times V \times t)$ (23).

RESULTS

The *frl* gene cluster is essential for Amadori product degradation. As we cannot exclude the possibility that *B. subtilis* possesses enzymes other than those encoded by the *frl* operon capable of degrading fructosamines, the physiological significance was investigated by generating a Δ*frlBONMDR* mutant. The growth and Amadori product consumption of the deletion mutant were compared with those of the wild-type strain (Fig. 2A). The growth of the wild type in a medium with crude preparations of the Amadori product fructosyl-arginine (containing residua of glucose and arginine) displays three phases. Glucose, arginine, and fructosyl-arginine were monitored by using enzymatic assay kits for glucose and Amadori products and UPLC analysis for arginine. The initial growth phase resulted from glucose consumption followed by arginine consumption, and finally the Amadori product was consumed. The lack of the last growth phase in the deletion mutant is consistent with the remaining constant concentration of Amadori products and demonstrates the inability of the Δ*frlBONMDR* strain to use fructosylamine. Thus, the genes of the *frlBONMDR* cluster appear to be instrumental in and necessary for Amadori product degradation. As the Δ*frlR* mutant degraded the fructosamines (see Fig. S1 of the supplemental material), *frlR* is not necessary for growth on Amadori products, whereas some or all of the genes of the operon appear to be necessary.

FrlR is a negative regulator of the *frlBONMD* operon. Results of reporter gene assays performed with a P_{frlB}-*lacZ* fusion integrated into the *amyE* locus (strain DE11; Fig. 2B) demonstrate a strong increase in expression from P_{frlB} during the growth on Amadori products. As outlined in the introductory remarks, the expression of the *frl* genes driven by P_{frlB} is under the control of the global regulator CodY, which represses transcription from P_{frlB} during growth on glucose (3). Based on the results outlined in Fig. 2, another transcriptional regulator besides CodY may participate; thus, we checked the possible but hitherto unknown involvement of the putative regulator FrlR, which is encoded adjacent to the structural *frlBONMD* genes (see the schematic representation in Fig. 1).

DE12 and DE13 (FrlR and CodY deletion strains, respectively) were constructed by replacing *frlR* and *codY* with erythromycin and kanamycin resistance genes, respectively (the double mutant was DE14) and the expression of the P_{frlB}-*lacZ* fusion was compared to the wild-type expression. Results obtained with the Δ*codY* strain, DE13, agree with those of Belitsky and Sonenshein (3), as the mutant displayed constant high-level β-galactosidase activities (Fig. 3A). Consistently, in our Amadori product medium, DE13 displayed constant high-level reporter activities already in the first growth phase (on residual glucose); however, the activity levels doubled in second growth phase (data not shown). The reporter gene assay of the *frlR* deletion strain (DE12) disclosed a similar strong repression of P_{frlB} by FrlR (Fig. 3A). The *frlR* mutation enhanced the β-galactosidase activity approximately 50-fold during the exponential growth phase and also 7-fold in stationary phase when grown in glucose medium. Such repression is at least partially independent of CodY, as the double mutant DE14 (Δ*frlR* Δ*codY*) was further derepressed in the stationary growth phase compared to the single mutants (about 100-fold; Fig. 3A).

The direct binding of FrlR to P_{frlB} was investigated by performing electrophoretic mobility shift assays (EMSAs). The entire intergenic region (199 bp) upstream of *frlB* was incubated with various concentrations of the FrlR protein, which was heterologously expressed in *E. coli* with a His tag for ease of purification and of immunological detection. In PAGE, a distinct shift of the promoter fragment upon the addition of 600 nM FrlR became visible (Fig. 3B).

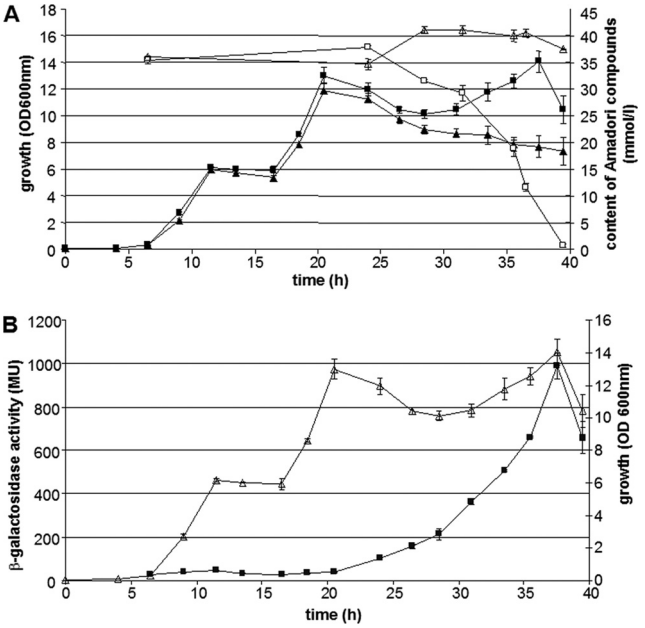

FIG. 2. Growth of *B. subtilis* in M9 Amadori product medium. (A) Comparison of the growth profiles of wild type (squares) and DE01 (Δ*frlBONMD* Δ*frlR*; triangles). The OD_{600}s were determined as a parameter for growth (filled squares and triangles), and the relative concentrations of Amadori products in culture supernatant (open squares and triangles) were quantified by use of GlyPro reagents (Genzyme) relative to a glycated hemoglobin calibrator control. (B) The expression of the *lacZ* reporter gene driven by P_{frlB} of wild-type strain DE11 (filled squares) during growth in M9-Amadori product medium (open triangles). Standard deviations are shown as error bars.

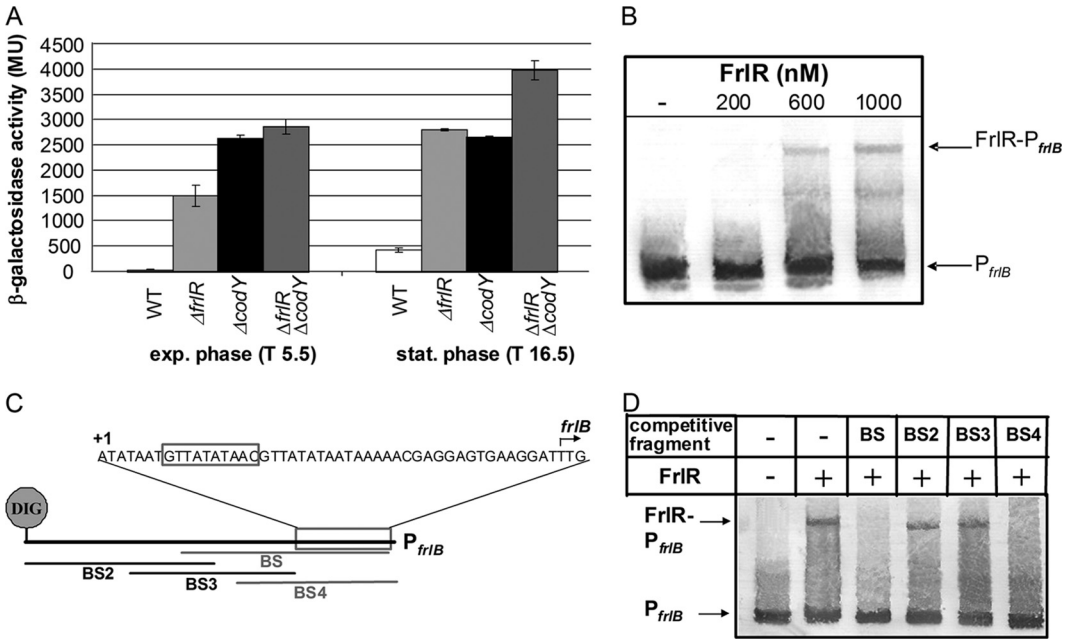

FIG. 3. (A) Measurement of the *frlB* promoter activity in the strains DE11 (wild type [WT]), DE12 (Δ*frlR*), DE13 (Δ*codY*), and DE14 (Δ*frlR* Δ*codY*), quantified as *lacZ* reporter gene activities in Miller units. Cells were grown in M9-Glc-NH_4 medium. Samples were taken at 5.5 h after inoculation ($T_{5.5}$; exponential [exp.] growth phase) and $T_{16.5}$ (stationary [stat.] phase). Levels for DE11 (WT) kept constant until the culture reached the stationary phase; maximum activity in the stationary phase is given in the figure and kept at this level until lysis occurred. Standard deviations are shown as error bars. (B) Analysis of binding of FrlR to P_{frlB} by EMSA. A volume of 0.5 nM 5′-labeled P_{frlB} was incubated without (−) or with 200 to 1,000 nM FrlRcHis. (C) Schematic illustration of applied unlabeled fragments of the *frlB* promoter for competitive EMSA experiments. The consensus GntR binding site is boxed, and the transcriptional start site (+1) is indicated as such (3). The CodY binding site (3) is located in fragment BS3. (D) Electrophoretic mobility shift assay with 5′-DIG-labeled P_{frlB} and different single competitor fragments in 20-fold excess. The amount of unlabeled competitors added to each reaction mixture was 10 nM (20× relative to the labeled P_{frlB}). The amount of 5′-DIG-labeled probe P_{frlB} was 0.5 nM. The concentration of FrlRcHis was 800 nM.

In order to localize the binding site of FrlR within the *frlB* promoter sequence, competitive mobility shift assays were performed. Four partially overlapping fragments of the *frlB* promoter were generated (Fig. 3C; see Fig. S4 in the supplemental material) and individually incubated in 20-fold excess with the complete labeled promoter fragment and the FrlR protein. Binding of FrlR to the competing fragments reduces the binding of FrlR to the labeled promoter fragment and, thus, decreases the amount of the shifted EMSA fragment. As shown in Fig. 3D, the decrease of the shifted fragment occurs with competitor DNA fragments BS and BS4 (Fig. S4), thus localizing the FrlR binding site to the overlapping regions of the two fragments. Within the identified region, GntR regulator type consensus sequence 5′-(N)yGT(N)xAC(N)y-3′ (where y is the number and N is the nature of the nucleotides being palindromic around the conserved GT-AC which flank several A/T bases [x]) is recognizable. In the *frlB* promoter region, a 26-bp palindrome with a central GTtaTAtaAC sequence (with capital letters representing conserved nucleotides) suggests that FrlR belongs to the HutC subfamily of the GntRs, as these are characterized by a central TA between the conserved GT and AC bases (30).

Expression of *frlR* is subject to autoregulation. To test possible autoregulation, a strain carrying P_{frlR} upstream of the *lacZ* gene was constructed. The expression of the reporter gene in the wild-type background (DE16) was rather low (~3 to 10 MU, depending on the growth phase and the medium). To further examine potential autoregulation, which is not uncommon for GntR regulators (31), the strain carrying the *frlR* deletion was examined. The P_{frlR}-*lacZ* expression in the Δ*frlR* strain was increased about 5- to 10-fold compared to that of the wild type (Fig. 4A). Subsequently, the direct binding of FrlR to its own promoter was tested by mobility shift assays. The shift of the promoter fragment upon the addition of 400 nM FrlR repressor (Fig. 4B) revealed the binding of FrlR, which agrees with negative transcriptional autoregulation.

The first intergenic region of the operon mediates the decrease in expression of the downstream genes. In qPCR analyses, *atpA*, a constantly expressed housekeeping gene, was used as a reference. The PCR analyses validated the reporter gene assays described above; however, though the two genes belong to the same transcription unit, expression of *frlB* was ca. 4-fold higher than that of *frlD* (data not shown). This agrees with recent tiling arrays in which expression was found to fade downstream of *frlB* (27). Hence, our attention was drawn to the first intergenic sequence ($I_{frlB\text{-}frlO}$) of the operon. We identified a perfect 38-bp-spanning palindrome (Fig. 1) which may form a hairpin with a stem of at least 17 bp, allowing for a 4-bp loop, possibly acting as pausing site for the RNA polymerase. Furthermore, the sequence in the center of the palindrome matches the FrlR binding site GT..AT..AC (Fig. 1), suggesting that FrlR has in addition to the *frlB* and *frlR* promoters a third binding site within the cluster. Indeed, EMSA experiments confirmed binding of FrlR to the intergenic region, as a shift

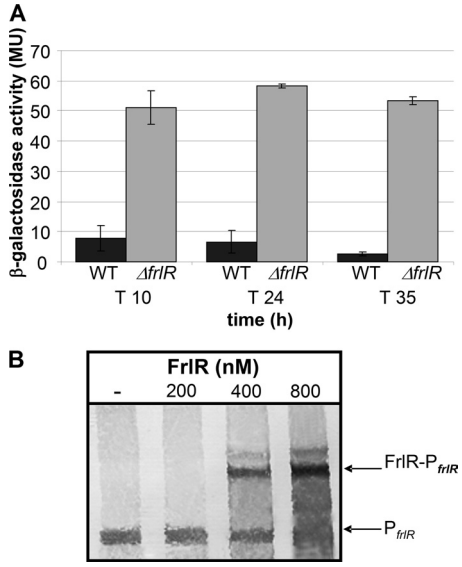

FIG. 4. Investigation of autoregulation of *frlR* expression. (A) The *frlR* promoter activities in strains DE16 (wild type) and DE21 (Δ*frlR*) were determined as *lacZ* reporter gene activities in Miller units. Cells were grown in M9-Amadori product medium, and samples were taken at three different time points. Standard deviations are shown as error bars. (B) Examination of FrlR binding to P_{frlR} by EMSA was carried out with 0.5 nM 5′-DIG-labeled P_{frlR} and various concentrations of FrlRcHis (200 to 800 nM).

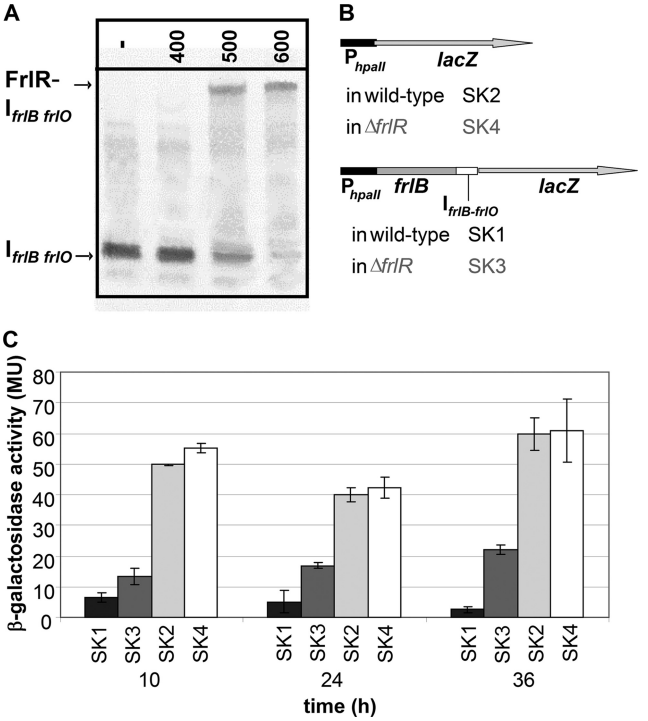

FIG. 5. The *frlB-frlO* intergenic region ($I_{frlB\text{-}frlO}$) decreases the expression downstream of *frlB*. (A) Analysis of the binding of FrlR to $I_{frlB\text{-}frlO}$ by EMSA. A 0.5 nM concentration of 5′-DIG-labeled $I_{frlB\text{-}frlO}$ was incubated without (−) or with 400 to 600 nM FrlRcHis. (B) Schematic illustration of the reporter gene constructs. The FrlR-independent *hpaII* promoter was cloned in front of the *lacZ* reporter gene in wild-type (SK2) and Δ*frlR* (SK4) constructs. Further constructs with the *frlB* gene and $I_{frlB\text{-}frlO}$ downstream of P_{hpaII} followed by *lacZ* (SK1 [wild type] and SK3 [Δ*frlR*]) were generated. (C) Measurement of promoter activity by β-galactosidase assay. Strains were cultivated in M9-Amadori medium, and at the indicated time points, the β-galactosidase activity was measured. Standard deviations are shown as error bars.

was seen at the addition of 500 nM FrlR to the labeled $I_{frlB\text{-}frlO}$ (Fig. 5A).

Promoter test studies were carried out to examine a possible expression reduction by the palindrome. The *frlB* gene with the intergenic region $I_{frlB\text{-}frlO}$ was cloned between P_{frlB} and *lacZ* to compare its expression to the expression of P_{frlB} alone. Such a reporter gene construct resembles the native situation, as the start codon of *lacZ* replaces the *frlO* start codon. In the wild type, the decreasing effect of the elongated construct with the intergenic region downstream of P_{frlB} was about 2- to 5-fold in comparison to the P_{frlB} alone (see Fig. S2 in the supplemental material). Expression from the P_{frlB} promoter in the Δ*frlR* background was much stronger than its expression in the wild type; a direct comparison with the wild type is not possible, as FrlR binds to both P_{frlB} and $I_{frlB\text{-}frlO}$.

This binding is why an FrlR-independent promoter was subsequently used to exclude overlapping effects by FrlR binding to the promoter and the intergenic region. The promoter P_{hpaII} of the *Staphylococcus aureus* pUB110 plasmid was cloned upstream of either the *lacZ* reporter gene only or the *frlB* gene, with $I_{frlB\text{-}frlO}$ also placed in between P_{hpaII} and *lacZ*. A schematic representation of the constructs is depicted in Fig. 5B. Reporter gene expression of these constructs in the wild type and the Δ*frlR* background was subsequently inspected (Fig. 5C). Though we cannot exclude the possibility that different junctions between the promoter and *lacZ* may influence expression of the reporter gene, it is unquestionable that FrlR reduces this expression. Furthermore, the results obtained with constructs resembling the native genetic organization are supportive of our conclusion, that the decreasing effect of the intergenic region is apparent and clearly not dependent on the upstream promoter, as the effects on expression with P_{hpaII} and P_{frlB} are similar. However, a considerable difference became obvious by using the P_{hpaII} promoter when wild-type and Δ*frlR* strains were compared (Fig. 5C), as the decreasing effect is stronger in the wild type, thereby suggesting that FrlR is involved in enforcing the reductive effect on expression.

DISCUSSION

Since the *B. subtilis* deletion mutant lacking the *frlBONMDR* cluster failed to consume and grow on fructosamines, the genes of the operon are not only mandatory but evidently also sufficient for the consumption of Amadori products. Moreover, fructosamines can apparently serve as the sole carbon and nitrogen sources in *B. subtilis* as for *E. coli* (40).

Our findings that the expression level increased at the end of the exponential growth phase/beginning of the stationary phase even in media with glucose as the sole carbon source agree with previous studies in which the global transcriptional regulator CodY was shown to downregulate the expression of the *frl* operon during exponential growth (3). However, compared to the corresponding single mutants, the Δ*frlR* Δ*codY*

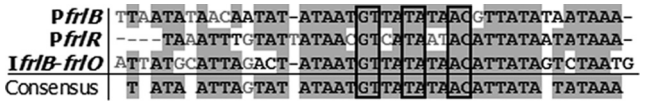

FIG. 6. Alignment of the identified FrlR binding sites in the *frlB* promoter, the *frlR* promoter, and the *frlB-frlO* intergenic region. Homologous bases are indicated as black letters on a gray background. The consensus motif for GntR regulators of the HutC family is framed in black.

double mutant displayed a clear increase in expression driven by P_{frlB} even without Amadori products being present in the medium, which suggested that FrlR acts as an additional repressor besides CodY, which has a known role. Moreover, the derepression in media supplemented with Amadori products was greater than that in glucose medium (16-fold in glucose medium versus 33-fold in fructosamine medium). Thus, in the wild-type strain, expression is almost totally repressed during exponential growth on glucose and partially derepressed after the exponential growth; however, full derepression requires the presence of Amadori products. During growth on arginine, repression of the wild-type strain is constant and on a maximum level even at the stationary phase. Under the presumption that fructosamines serve as effector molecules, the strong derepression during growth on fructosamines could be understood. However, Amadori products may not be the only effectors, since during growth on glucose, half-maximal derepression (16-fold in comparison with 33-fold) was demonstrated. Interestingly enough, within the crystal structures of TreR, YvoA, and FrlR, there is a sulfate ion at the same location, which is characterized by conserved residues for sulfate binding (28). For TreR, it has been proposed that sulfate mimics the phosphoryl group of the effector trehalose 6-phosphate (29), and YvoA is inactivated by its cognate effector *N*-acetylglucosamine-6-phosphate (28).

This agrees with the finding that intracellular glycation frequently occurs in *E. coli*, baker's yeast, and mammals (6, 13, 24, 25, 35, 36). Moreover, the glycating potential of glucose 6-phosphate is 3- to 20-fold higher (depending on the amine) than that of glucose (11, 13, 35, 36). Hence, fructosamine 6-phosphate, rather than fructosamine, is likely to act as the actual (or as an additional) effector.

An alignment of the FrlR binding sites (Fig. 6) revealed the conserved operator motif (GT..TA..AC) of the HutC subfamily of GntR regulators (30) flanked by conserved palindromic sequences of different lengths, and indeed, preliminary mutation studies (with replacements of the conserved G and C with A and T within the *frlB* promoter) revealed a partial loss of FrlR binding in EMSA experiments and reporter gene assays (see Fig. S3 in the supplemental material). The deletion of *frlR* did not lead to a noticeable phenotype, but overexpression eventually was lethal (data not shown), possibly indicating that FrlR is involved in the regulation of other loci, though, due to the resulting large amount of the protein during overexpression, the repressor may also switch off promoters with binding sites displaying a lower affinity. As for *E. coli*, the glycopeptidase Gcp may be a putative target for FrlR regulation. The enzyme may protect the bacteria from damage caused by Amadori-modified proteins, and indeed, highly conserved Gcp proteins are seen in almost all sequenced organisms (21).

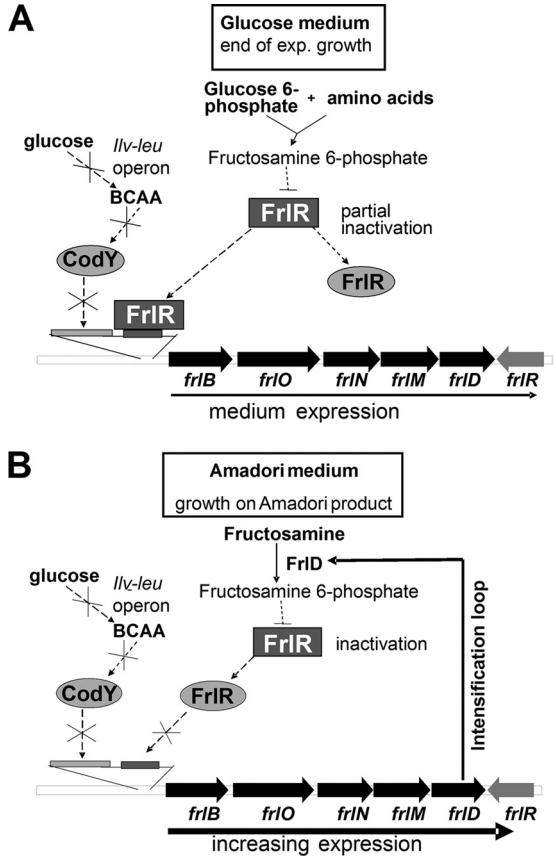

FIG. 7. Postulation of the regulation of the *frl* genes in *B. subtilis*. (A) During growth on glucose, intracellular glucose 6-phosphate leads to the glycation of proteins and amino acids; such fructosamine 6-phosphates partially inactivate FrlR, thereby promoting expression of the *frl* operon. CodY is inactive at the end of the exponential growth phase, due to glucose exhaustion. During growth on glucose, the CcpA-mediated activation of the *ilv-leu* operon causes the synthesis of the branched-chain amino acids (BCAA) isoleucine, leucine, and valine acting as CodY activators. (B) At the beginning of growth on Amadori products, import and phosphorylation of the fructosamines (by FrlD) represent the limiting step, since the *frl* operon is initially expressed at a low level. The more Amadori products are imported and phosphorylated (with the help of increasing expression of *frlONMD*), the more FrlR is inactivated, resulting in the further increase of expression, initiating an intensification loop and allowing for positive feedback regulation.

As there was an additive effect of the palindrome and FrlR binding in our reporter studies, we assume that the palindrome may form a hairpin, causing transcription termination; additionally, binding of FrlR may build a roadblock for the RNA polymerase, thereby weakening expression of the downstream genes (27).

Such downregulation of the transporter genes *frlONM* and of the kinase gene *frlD* makes sense, taking into account, on one hand, kinetic parameters of both the kinase and the deglycase, with the former displaying considerably higher k_{cat} and k_{cat}/K_m values for all tested substrates than FrlB (40). On the other hand, glucose 6-phosphate actually produces significant intracellular glycation, and solely FrlB is needed for their degradation. The transporter encoded by *frlONM* is probably necessary for the import of fructosamines; thus, expression of the

whole operon is required for growth on Amadori products. The *frl* genes in *B. subtilis* apparently have two physiological functions: facilitating growth on extracellular Amadori products and protecting cells from damage by removing intracellularly formed fructosamine phosphates.

As outlined in Fig. 7, intracellular glucose 6-phosphate may result in glycation of proteins and amino acids (Fig. 7A). Fructosamine 6-phosphates may inactivate FrlR, eventually leading to increased expression of the *frl* operon. CodY is inactive at the end of the exponential growth phase when glucose is exhausted. During growth on glucose, the CcpA-mediated activation of the *ilv-leu* operon causes the synthesis of the branched-chain amino acids isoleucine, leucine, and valine acting as activators for CodY.

On Amadori products, the import and phosphorylation of the fructosamines (by FrlONM and FrlD, respectively) may be the rate-limiting step, as the *frl* operon is initially expressed at a low level. The more Amadori products are being imported and phosphorylated (with the help of increasing expression of *frlONMD*), the more FrlR is inactivated eventually leading to increased expression and, thus, to an intensification loop, i.e., positive feedback regulation (Fig. 7B).

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